

Pollen Grains Induce a Rapid and Biphasic Eczematous Immune Response in Atopic Eczema Patients

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Key Words

Pollen • Atopic eczema • Nickel • Contact dermatitis

Abstract

Introduction: Eczematous reactions to type I allergy-inducing antigens are documented in a subgroup of patients with atopic eczema. Yet, the underlying immunological mechanisms are not well understood. **Material and Methods:** To delineate the effect of native pollen grains on human skin of healthy and atopic individuals we performed patch tests (atopy patch test with native pollen grains, PPT). Nickel patch tests (NPT) served as an established model of contact dermatitis. Skin site biopsies were taken 6–96 h after allergen application and investigated immunohistochemically. **Results:** Histology of positive patch tests showed an influx of mononuclear cells (predominantly CD4+, CD25+, CD45RO+). This influx was detected earlier in the PPT reaction than in the immune response to nickel. A biphasic cytokine response could be detected in the PPT: IL-5 dominated in the early,

IFN- γ in the late phase. The NPT was continuously dominated by IFN- γ . Dendritic cell subpopulations imitated the earlier kinetics of the mononuclear infiltrate. **Discussion:** Thus, pollen grains induce eczematous reactions in susceptible individuals. This reaction appears clinically and immunohistochemically similar to the contact hypersensitivity reaction to nickel but follows a faster kinetic and a biphasic course: Th2 and IgE in the early (24 h) and Th1 predominance in the late (96 h) phase.

Introduction

Atopic eczema (AE) [1] is a chronic relapsing inflammatory skin disease which is often associated with asthma and/or hay fever and a positive family history of atopy. Its main features are eczematous skin lesions with a typical, age-related distribution and severe pruritus [2, 3]. It is a disease caused by combined influences of genetic and environmental factors [4] leading to a Th2-dominated immune response against various allergens [5]. Skin irritants, systemic or local infections, environmental pollutants, stress and hormonal changes may play an aggra-

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vating role in the pathophysiology of AE [6, 7]. Especially aeroallergens may induce eczema flares [8, 9].

Current concepts follow the idea that allergens are able to penetrate the barrier-disturbed epidermis [10] where they are taken up by Langerhans cells which migrate to the regional lymph nodes and present the novel antigen to naïve T cells [11]. The relationship between antigen exposure via pollen grains and disease activity was already demonstrated as early as 1949 [12]. Notably, most recently a population-based panel study demonstrated that outdoor grass pollen exposure significantly exacerbated symptoms in children with eczema, especially in those sensitized against grass pollen [13]. These studies suggest that allergens which are known to induce type I allergy with the typical Th2-dominated immune response and production of specific IgE antibodies are also involved in the elicitation of eczematous reactions. The atopy patch test (APT) has been used as a model for early AE lesions [14, 15] and is performed with protein extracts of the respective allergen carrier, e.g. house dust mite or pollen [16, 17]. The standardized APT technique has been developed in a series of multicentre trials and is regarded to be a suitable and reproducible procedure for evaluating the clinical relevance of IgE sensitization for eczematous skin lesions [18–20]. Since pollen themselves release not only allergens, but are rather a package of myriads of substances including the recently described proinflammatory and immunomodulatory lipid mediators (pollen-associated lipid mediators – PALMs) [21–24], we exposed our patients and controls to native pollen grains.

The present study aims at analyzing an eczematous reaction to classically type I, IgE-mediated allergy-inducing agents. Since an eczematous reaction is traditionally contributed to type IV hypersensitivity-inducing agents we took the nickel patch tests (NPT) as reference inflammatory skin reaction in order to classify pollen eczematous reaction in the traditional model of types of hypersensitivity reactions to environmental substances.

Materials and Methods

Patients and Subjects

For the analysis of the time course of an acute eczematous reaction two different main groups were enrolled into the study following the declaration of Helsinki protocols after written informed consent had been given. (1) Patients with AE (n = 6) diagnosed according to the criteria of Hanifin and Rajka [25] sensitized against grass and/or birch pollen (specific IgE class 3 or higher) and with a positive APT. Allergen-specific and total IgE levels in serum were determined by CAP-FEIA (Pharmacia, Uppsala, Sweden). (2) Patients with positive NPT (n = 5) but negative personal

and family history of AE or atopy. In addition, RAST and prick tests for 15 common allergens including birch and grass pollen were negative and total IgE was <20 IU/ml. (3) Healthy volunteers (n = 4) without history and without clinical signs of atopy or other allergic diseases. (4) Patients with AE (n = 4) and sensitizations to grass and birch pollen but a negative APT. Biopsies from long-lasting eczematous skin lesions (1 month) of an AE patient served as reference for a chronic lesion. None of the patients had received systemic steroids, antihistamines or UV light to the test site for at least 14 days prior to testing. The ethical committee of the Technical University of Munich approved the study.

Pollen Patch Test and NPT

Pollen patch tests (PPT) were performed using the standardized procedure of APT [18] with 1 mg intact pollen grains instead of pollen extracts. Commercially available grass (*Phleum pratense* L.) and/or birch (*Betula alba* L.) pollen (Allergon, Ängelholm, Sweden) were applied in large Finn chambers (11 mm in diameter) on the back of the patients using petrolatum as a carrier. Petrolatum alone served as a negative control. After 48 h the Finn chambers were removed and the tested areas were marked. The test was evaluated after 48 and 72 h. Positive reactions were classified according to the European Task Force on Atopic Dermatitis (ETFAD) 2000 reading key [8]. Epicutaneous provocation with nickel was performed using petrolatum containing 5% NiSO₄ [nickel(II)-sulfate 5%, Hermal, Reinbeck, Germany] applied on the patients' back for 48 h. The reactions were classified using the criteria of the International Contact Dermatitis Research Group and ranged between 1 and 3, according to the state of inflammation.

Tissue Processing and Staining

Punch biopsy specimens (5 mm in diameter) were taken under local anesthesia (1% lidocaine) from the centre of the patch test areas at various time points (6, 24, 48, 72, 96 h after nickel/pollen application). Each patch test field was biopsied only once. Biopsies were immediately frozen at –70°C and stored until further handling.

From these biopsies, 4-µm-thick sagittal cryostat sections were performed on a freezing microtome (cryostat Leitz 1720, Wetzlar, Germany) and mounted on glass slides coated with poly-L-Lysine (Sigma, Munich, Germany). The slides were air-dried for 2 h and then fixed for 10 min in dry acetone (Sigma). Two slides of all specimens were stained with haematoxylin-eosin. The immunohistochemical staining was performed in part in the 'Tech Mate Horizon' (Dako, Hamburg, Germany) (table 1) according to the APAAP method.

Some stainings were performed manually (table 1): the sections were fixed with 3% buffered paraformaldehyde (Sigma) for 20 min, washed in Trizma base buffer (Sigma) containing 1% bovine serum albumin (Sigma) for 10 min and then incubated with the primary antibody diluted in antibody diluent (Dako, Hamburg, Germany) for 1 h. After washing for 10 min, the secondary antibody was added for 25 min. After this step and repeated washing, alkaline phosphatase was added for 25 min. The reactivity of this enzyme was visualized using the super sensitive detection kit (Bio Genex, San Ramon, Calif., USA). The activity of the alkaline phosphatase was inhibited by levamisole (Dako, Hamburg, Germany). Finally, all sections were washed again, slightly counterstained with haemalaun, washed and mounted in gelatine (Merck, Haar, Germany).

Table 1. Characterization of antibodies used for immunostaining

Antibodies	Isotype	Clone	Dilution	Source
CD1a ¹	IgG2a	NA1/34	1:50	Dako, Hamburg, Germany
CD3 ¹	polyclonal	polyclonal	prediluted	Dako, Hamburg, Germany
CD4 ¹	IgG1	EDU-2	1:50	NovoCastr, Dossenheim, Germany
CD8 ¹	IgG1	C8/144B	prediluted	Dako, Hamburg, Germany
CD25 ¹	IgG1	ACT-1	1:100	Dako, Hamburg, Germany
CD45RO ¹	IgG2a	UCHL-1	prediluted	Dako, Hamburg, Germany
BDCA22	IgG1	AC144	AC144	Miltenyi Biotec, Bergisch Gladbach, Germany
CD1b ²	IgG1	IOT6b	1:100	Immunotech, Marseille, France
FcεRI ²	29C6	IgG1	1:500	J. Hakimi and R. Chizzonite, Hoffmann-La Roche, Nutley, N.J., USA
CD64 ¹	IgG1	10.1	1:100	BD Pharmingen, Heidelberg, Germany
CD68 ¹	IgG2b	Y1/82A	1:200	BD Pharmingen, Heidelberg, Germany
IFN-γ ²	IgG1	45.15	1:50	Alexis, Grünberg, Germany
IL-5 ²	IgG1	9906.1	1:300	R&D, Minneapolis, Minn., USA
IgE ²	IgG1	CIA-E-7.12	1:300	Dako, Hamburg, Germany

¹ Antibody was stained with the automated immunostainer, TechMate Horizon.

² Antibody was stained manually.

Light Microscopy: Quantification and Statistical Analysis

Microscopic investigation was performed with the Axioplan2 (Zeiss, Jena, Germany) light microscope. Immunohistochemical results were evaluated using the software programme 'KS300' developed by Zeiss which calculates the relative positive area in percent and the absolute positive area in square micrometres by measuring colour intensity. Quantification of two slides per stain was performed at 500× magnification in a blinded manner by two independent analyzers. Data from both analyzers was pooled. Quantification of the colour intensity was performed for dermis and epidermis. Since BDCA2 positivity was too low for a reliable online quantification, all BDCA2+ cells in a defined area (dermis and epidermis separately) were counted at 500× magnification by two independent investigators. The area of interest was defined using the software KS300. With these data, the number of cells per square millimetres was calculated for each biopsy.

Student's unpaired t test was used to compare the reactions of APT versus NPT and control groups. A p value <0.05 was considered significant.

Results

Clinical Aspects of Patch Test Reactivity

Native pollen grains induced acute eczematous reactions in patients with AE and known positive APT to pollen protein extracts 24–48 h after allergen application (data not shown). The test site showed an oedematous erythema with papules and seropapules resembling those seen in positive NPT. However, patients reacting to nick-

el showed a slower kinetics: eczematous reactions were seen after 48–72 h, while after 24 h none or only a slight erythema was observed. Notably, no acute eczematous reactions to native pollen grains were observed in AE patients with known sensitization against *P. pratense* or *B. alba* but negative APT in the past. None of our healthy controls showed a cutaneous reaction to native pollen grains. The petrolatum controls in all groups did not show any clinical signs of inflammation.

Histological Evaluation of Skin Biopsy Specimens

Hematoxylin-eosin-stained sections of biopsies taken from positive PPT and positive NPT showed a strong influx of mononuclear cells into dermis and epidermis. Acantholysis and intercellular oedema (spongiosis) were also observed in the epidermis. The strongest dermal and epidermal reaction was observed after 48 and 72 h for the PPT while the NPT showed a maximum after 72 and 96 h. After 96 h, the level of cellular infiltrate in the PPT was declining almost to the basic value, while in the NPT a strong infiltrate could still be observed (fig. 1a).

Immunohistochemical Characterization of the T Cell Infiltrate

Immunohistochemical analysis of the T cell infiltrate revealed that the majority of immigrating cells were CD3+CD4+ T cells both in NPT and PPT (fig. 1b, 2).

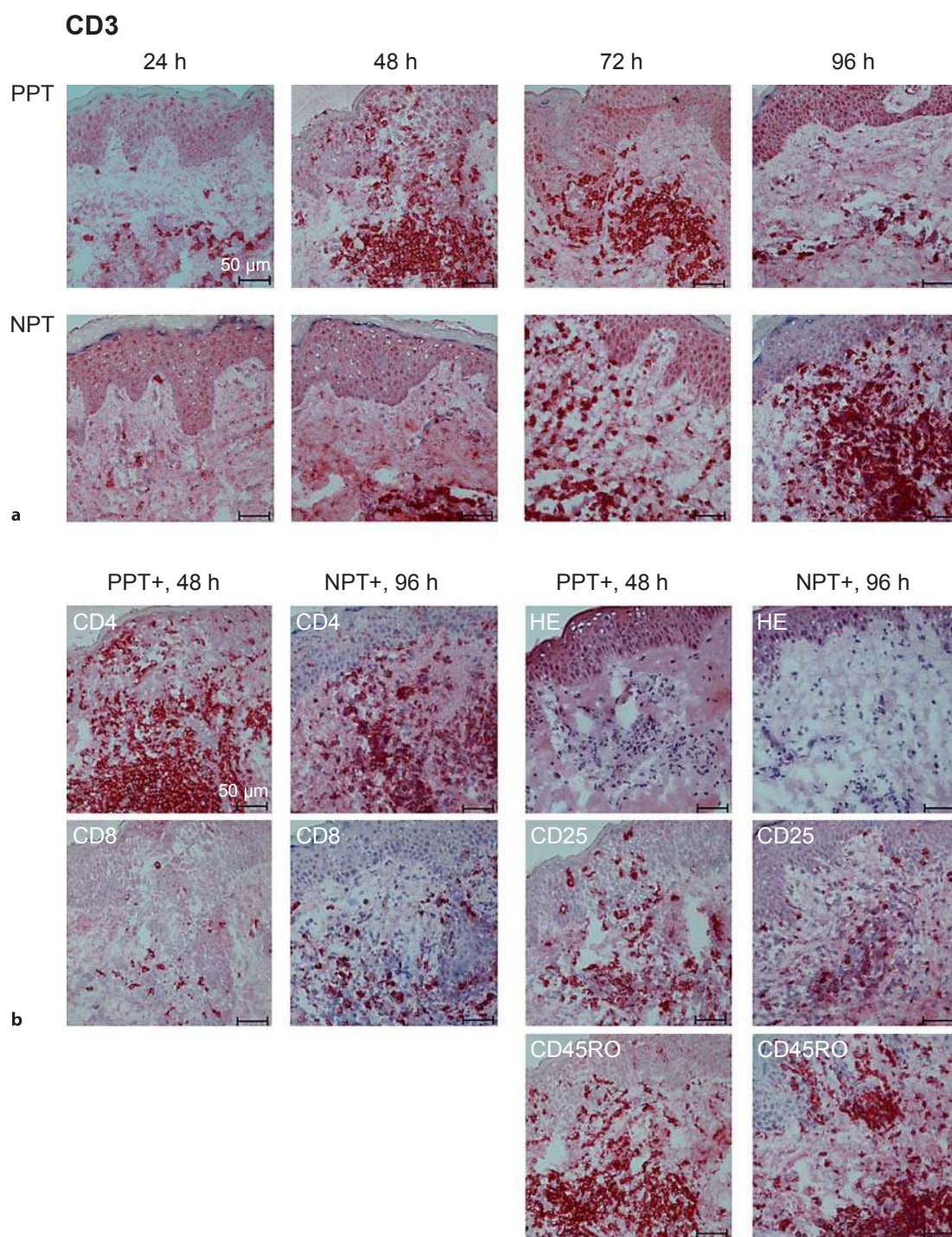
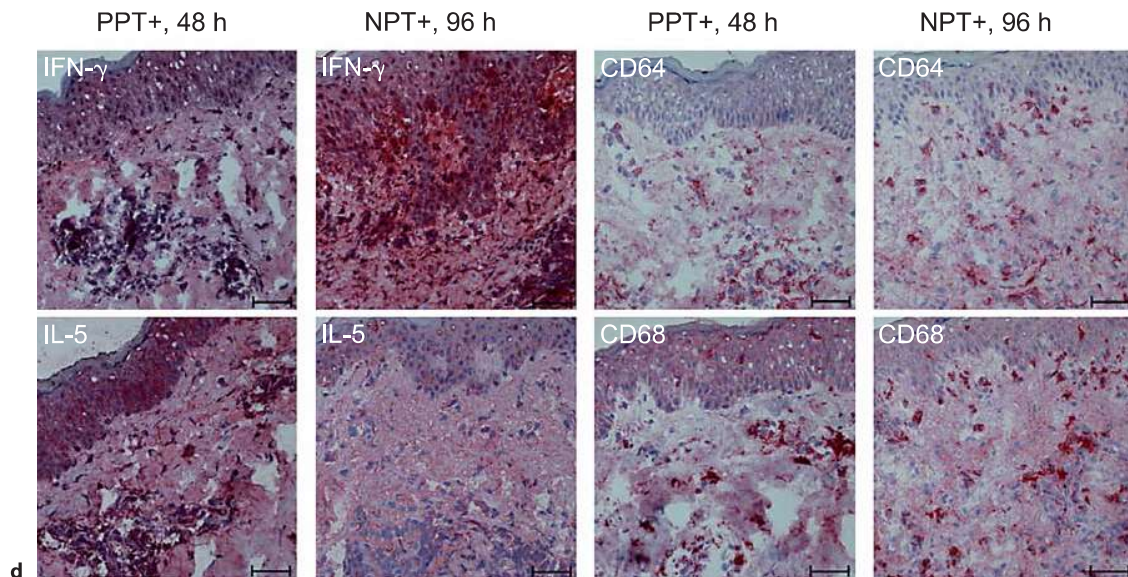
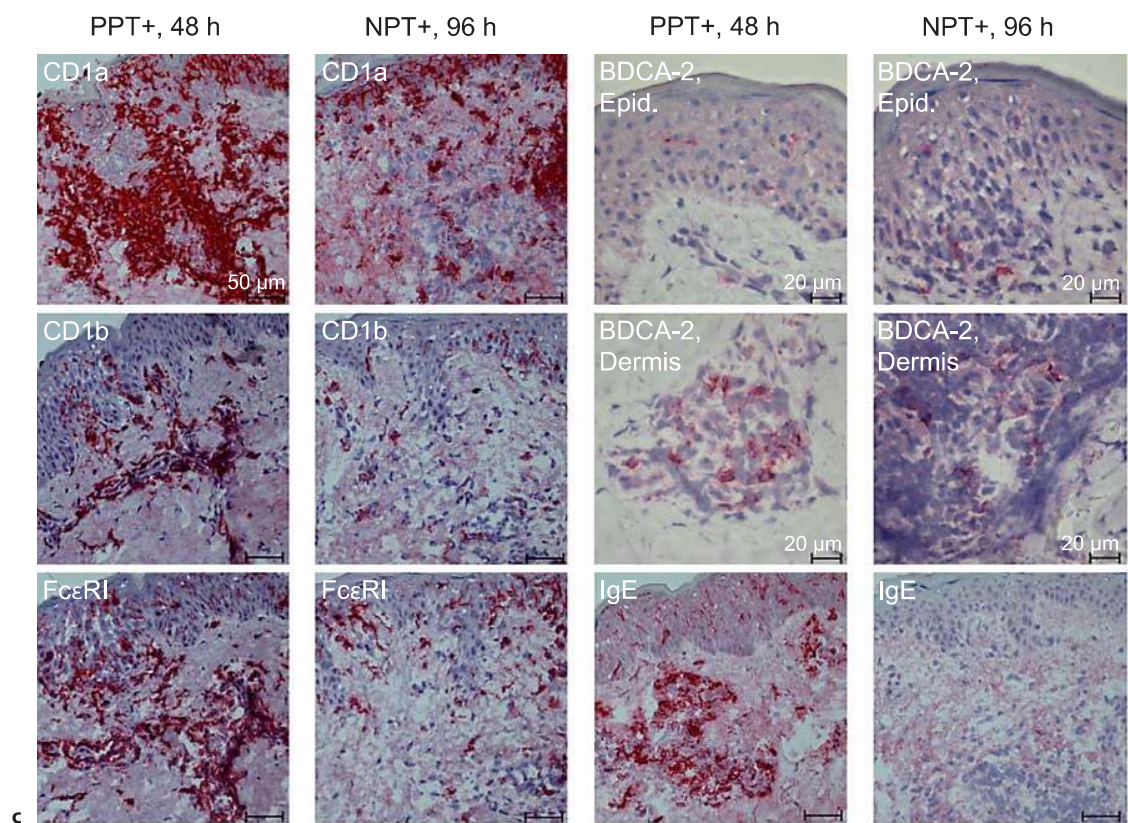


Fig. 1. Representative light micrographs of immunohistochemical stainings of PPT and NPT lesions. **a** The kinetics (24–96 h after allergen application) of the influx of CD3+ cells in PPT and NPT is shown for one patient each. **b** Characterization of the T cell infiltrate in PPT and NPT lesions (same patient as shown in **a**) at the climax of the immune reaction (PPT 48 h, NPT 96 h after allergen application). **c** Immunohistochemical analysis of DC markers and

IgE in NPT and PPT lesions, magnification for BDCA2 staining 800 \times , scale = 20 μ m. **d** Representative staining for the cytokines IFN- γ and IL-5 and monocyte/macrophage markers in PPT and NPT lesions at 48 and 96 h after allergen application. HE = Haematoxylin-eosin; Epid. = epidermis. **a–d** Magnification 400 \times (scale = 50 μ m) unless otherwise stated.



However, in APT the maximum influx of CD3+ T cells was lower compared to the NPT. An increase up to the late time point of 96 h of CD8+ cells was observed only in the NPT (fig. 2), while CD8+ cells in the PPT increased from 6 to 24 h and then remained on a level lower than that of the NPT until 96 h after allergen application. CD4+ T cells increased continuously until 72 h in the PPT. In the case of NPT, a slight elevation until 48 h and a strong peak after 72 h were observed. Both reactions declined after 96 h. Both NPT and PPT showed primarily an influx of CD45RO+ memory T cells. CD25+ cells showed a crescendo-decrescendo reaction both in NPT and PPT with a tendency – without significance ($p = 0.2$ for 48 h, $p = 0.1$ for 72 h) – of a higher percentage in the PPT reaction (fig. 1b, 2).

Analysis of Antigen-Presenting Markers and IgE

IgE positivity strongly increased in the positive PPT biopsies up to 24 h after pollen application and decreased continuously until 96 h but displayed still elevated levels compared to the other groups (fig. 1c, 3). IgE was found neither in the NPT+ biopsies nor in sections of healthy controls (fig. 3). FcεRI-bearing cells were present after 24 h only in the PPT sections. They peaked at 24 h and remained elevated up to 96 h. Both, the chronic AE lesion and the non-lesional skin of AE patients showed slightly elevated levels of FcεRI-bearing cells. Normal, healthy skin from the non-atopic patients was almost negative for FcεRI. The number of CD1a+ cells appeared higher in the PPT than in the NPT biopsies which was significant after 72 h ($p = 0.037$) (fig. 3). The chronic AE lesion showed a higher expression of CD1a in the epidermis (fig. 3). The most obvious difference between PPT and NPT occurred within the marker CD1b, staining primarily inflammatory dendritic epidermal cells (IDEC) [26], although overall positivity was low compared to CD1a. CD1b+ cells increased in PPT rapidly between 6 and 24 h, peaked at 24 h and remained at elevated levels (fig. 1b, 3). In contrast, in the NPT an increase in CD1b+ cells was only observed after 72 h. In order to investigate the occurrence of plasmacytoid dendritic cells (pDC), we stained the sections for BDCA2. However, the rate of pDCs in all sections was too low for automatic quantification. So they were counted manually in the dermis and in the epidermis (fig. 1c, 3). Even though pDCs were present in the epidermis of both PPT and NPT lesions the occurrence of pDC was much lower compared to the dermis (ratio approximately 1:5, depending on the different time points). Comparable to the IDECs, dermal pDCs peaked at 48 h in PPT biopsies. In NPT sections the highest num-

ber was observed after 72 h. CD64, Fcγ receptor I, expressed on macrophages and monocytes showed, as CD1b and BDCA2, a much faster kinetics in PPT compared to NPT (significant difference after 24 h, $p = 0.039$) while the rate of CD68+ cells appeared equally over the time course in PPT and NPT, being slightly higher at 24 h ($p = 0.013$) and 48 h ($p = 0.528$) in PPT sections but peaking at 72 h in both reactions (fig. 1d, 3). Notably, a faint positivity for the markers CD1b, CD64, CD68 and FcεRI was observed in the negative APT, both from patients with AE and healthy controls.

Cytokine Profile

Sections were also stained for interleukin-5 (IL-5) and interferon-γ (IFN-γ) in order to identify Th1- or Th2-dominated immune responses. Both cytokine antibodies – IL-5 and IFN-γ – showed typical cytoplasmic staining (fig. 1d). With regard to the T helper cell response, a clear dichotomy of the eczematous response to pollen in skin was observed while the NPT reaction was Th1 dominated over the entire time course of the eczematous reaction (fig. 4a, b). In the initiation phase of the eczematous reaction to pollen grains the IL-5 production was predominant over IFN-γ production. In the late and chronic phases the situation was reversed and IFN-γ production predominated over IL-5 production (fig. 4b). The biphasic course of the cytokine profile in PPT became most evident by the ratio of IFN-γ and IL-5 positivity in the cryosections (fig. 4a).

Discussion

Eczematous reactions caused by type I allergy-inducing antigens seem to play a role in maintaining and exacerbating AE, and yet the underlying mechanisms of this immune reaction are not well understood. In this study, we characterized the *in vivo* immune response to pollen grains immunohistochemically over a time course from 6 to 96 h after allergen application and compared it to the well-defined delayed-type hypersensitivity reaction to nickel. We found that both reactions were comparable in principle, but differed in three points: the PPT reaction to pollen followed a faster kinetics, the maximum intensity of the reaction was smaller and the infiltrating T cells showed a biphasic cytokine profile.

Concerning the T cell infiltrate, the maximum intensity of the reaction reflected by the amount of CD3+ T cells was higher in NPT than PPT sections, but the PPT reaction followed a faster kinetics. CD8+ T cells became

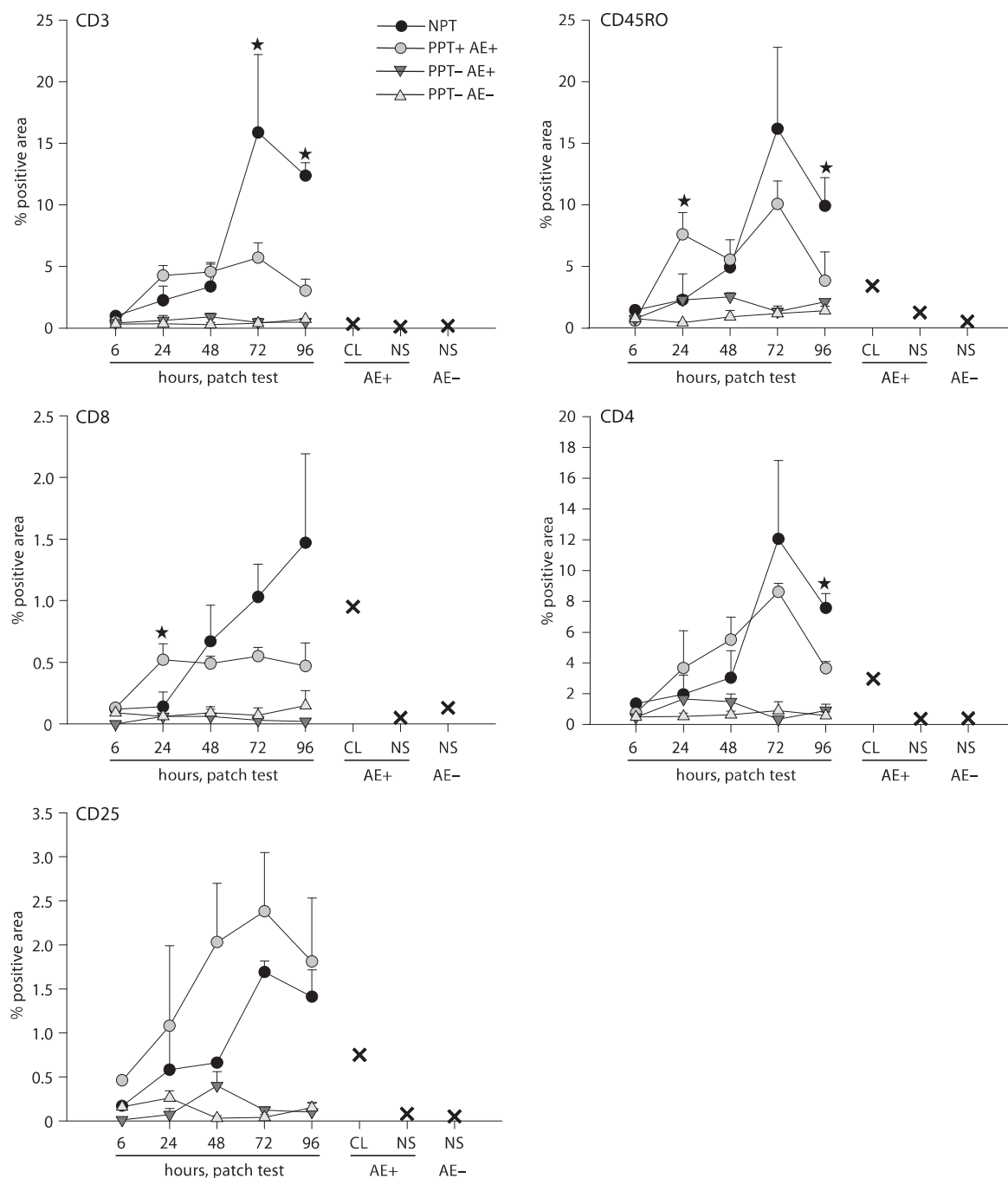
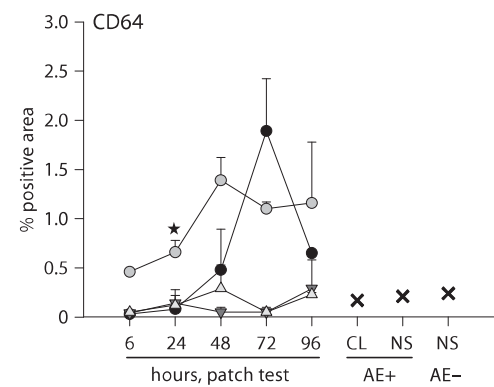
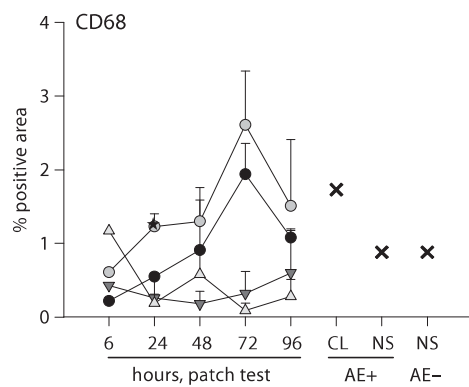
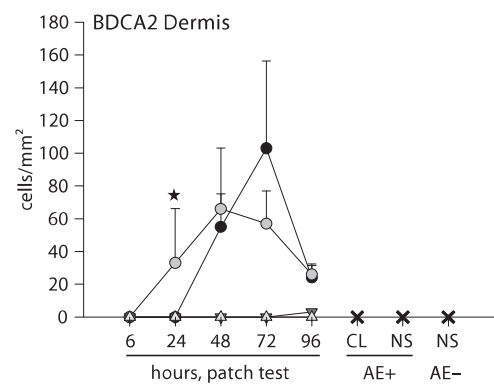
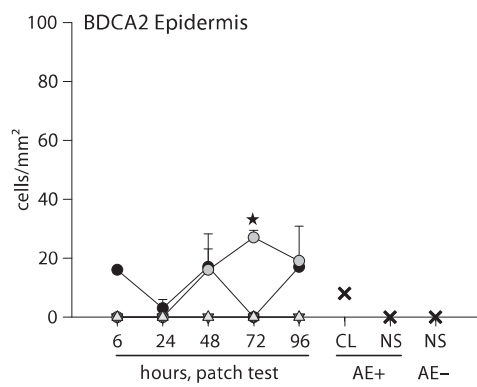
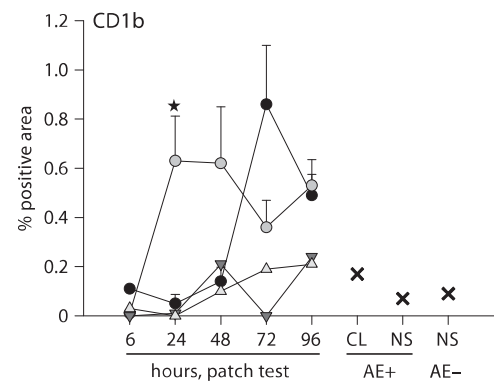
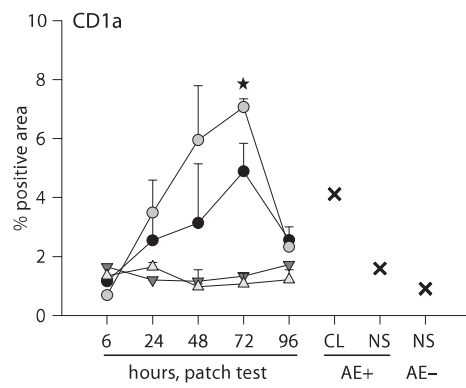
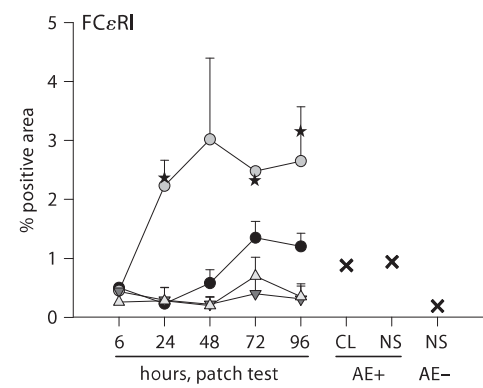
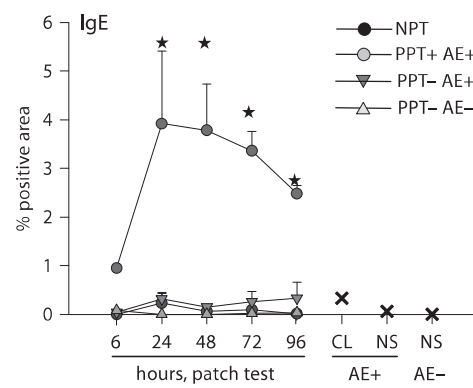


Fig. 2. Quantification of the T cell infiltrate in (PPT and NPT lesions: biopsies from PPT and NPT were taken 6–96 h after allergen application and stained for CD3, CD45RO, CD8, CD4 and CD25. Stained cryosections were quantified with the aid of the computer program KS300 (Zeiss, Jena, Germany). Mean \pm SEM of the percentage of the relative positive area of the respective antibody is shown from stained cryosections from 6 (positive PPT),

5 (positive NPT), and 4 (negative PPT in healthy donors and negative PPT in AE– patients) different donors. ★ = Significant ($p \geq 0.05$) differences between positive APT and NPT biopsies. Furthermore, the results of the chronic AE lesion (CL, $n = 2$), the non-lesional skin of atopic patients (NS AE+, $n = 2$) and normal skin of healthy volunteers (NS AE–, $n = 2$) are shown on the right of the diagrams.



more prominent in the NPT lesions especially at late time points (72 and 96 h) while they remained scarce in PPT sections. These results explain why a recent study investigating NPT described a much higher percentage of CD8+ when looking after 72 h [27]. At an earlier time point, our in situ results approximately correlate to the in vitro results from Werfel et al. [28] showing a proportion of 10–15% CD8+ allergen-specific T cells from lesional AE skin. Our results confirm previous reports [29, 30] demonstrating that more inflammatory cells are present in non-lesional AE skin compared to non-atopic skin, albeit in far smaller amounts than in lesional AE skin.

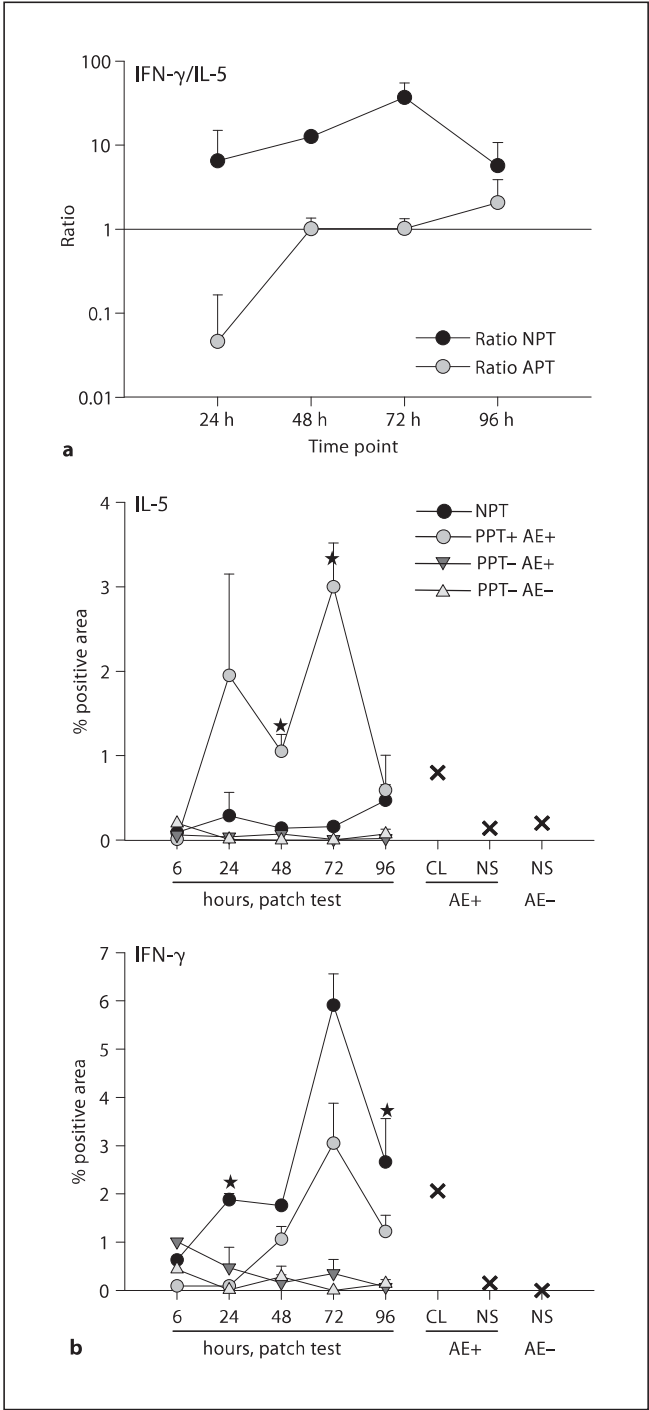
In both acute eczematous reactions (PPT and NPT) a predominance of memory T cells (CD45RO+) was obvious. Furthermore, we detected a slightly enhanced expression of CD25 in the PPT compared to the NPT, but since CD25 is also expressed on DC, this could in part be due to the higher expression of DC markers in PPT and AE (chronic lesion and healthy skin of AE patient) and the earlier influx of DCs in PPT lesions.

Cytokines produced by subsets of T cells may play a role in regulating some of the features of atopic allergic inflammation. Staining for IFN- γ and IL-5 revealed that at the early time points (24 h) IL-5 dominated over the expression of IFN- γ in the eczematous reaction to pollen grains while at the later time points (96 h) and in the chronic lesion IL-5 declined and an IFN- γ dominance was observed. Thus, our observations of a biphasic course of the cytokine profile in acute eczematous reactions to native pollen grains extend previous studies performed

on APT reactions to house dust mite [31, 32]. Taking our recent findings into account, the early Th2 response in the skin and the high IgE positivity in the PPT sections could in part be due to Th2-polarizing substances such

Fig. 3. Analysis of the expression of antigen-presenting cell markers and IgE in PPT and NPT lesions: cryosections of all donors (see legend of fig. 2) were analyzed for the staining for IgE, Fc ϵ RI, CD1a, CD1b (IDEC), BDCA2 (pDC in epidermis and dermis separately) and the macrophage and monocyte markers CD68 and CD64, respectively. Results are shown as mean values \pm SEM of positive staining area for the respective antibody quantified by KS300 computer software. ★ = Significant ($p \geq 0.05$) differences between positive PPT and NPT biopsies.

Fig. 4. Analysis of the cytokine profile in PPT and NPT lesions. **a** Ratio of the quantified relative positive area of IFN- γ and IL-5 after 24–96 h after allergen application in NPT and PPT biopsy cryosections. **b** Mean \pm SEM of the quantified relative positive area for IFN- γ and IL-5 in the time course of positive NPT ($n = 5$), APT ($n = 6$) and negative PPT (each $n = 4$) in AE patients and healthy controls. Furthermore, results from chronic AE lesion (CL, $n = 2$), non-lesional skin of atopic patients (NS AE+, $n = 2$) and normal skin of healthy volunteers (NS AE-, $n = 2$) are shown on the right. ★ = Significant ($p \geq 0.05$) differences between positive PPT and NPT biopsies.



as phytoprostanes released from pollen itself [23]. The results of NPT showing a Th1 pattern over the whole time course are in line with data from the literature [33].

At the same time as the change in cytokine profile in PPT, we observed a substantial influx of macrophages and monocytes (CD64+, CD68+ cells) at PPT sites. Recently, macrophage-produced IL-12 was shown to be able to induce Th1 development in vitro [34]. This suggests that the influx of macrophages might induce a change in local skin microenvironment favouring IFN- γ . Interestingly, in the PPT lesion the decrease in early peaking IgE is accompanied by the increase in CD64, CD68 and the switch to the Th1 domination.

The elicitation of contact dermatitis presumably requires close interactions between infiltrating T cells and antigen-presenting cells, which either reside in the skin or migrate from blood. Langerhans cells are the resident DC population in skin epidermis where they form a contiguous network to detect any invading pathogen or antigen [35]. The significantly higher number of CD1a-expressing cells in the skin of AE patients as shown in the present study is of special interest with regard to the recent publication showing that lipids from pollen are presented in a CD1-restricted manner [36, 37]. A second CD1a+ epidermal DC population, the so-called IDEC [38], has been described to be the relevant IgE-binding, Fc ϵ RI-expressing epidermal DC population in AE [39, 40]. In our study, CD1b, staining primarily IDECs, occurred in PPT as early as 24 h after pollen application while in the NPT an increase in CD1b positivity was observed earliest after 72 h. This marker thus correlates with the faster clinical manifestation of acute eczema in PPT. Furthermore, it underlines the central role of IDECs in the development of the eczematous reactions in the early phase [26]. In the chronic AE lesion CD1b+ cells were scarce, but still more highly expressed than in non-lesional and healthy skin. In contrast to that, CD1a was also highly expressed in chronic lesions suggesting that the DC alteration during skin lesion formation can be subdivided into early and late events, with the influx of IDECs as an early event and the predominance of CD1a+ cells as a late event.

pDC, also known as natural IFN- α/β -producing cells [41], have been described to be present in skin in contact dermatitis and psoriasis and in a lower number in AE [42], where their inactivity is associated with eczema herpeticum [43]. We observed an earlier influx of pDCs into the dermis in PPT (24 h) compared to the NPT (48 h). However, the percentage of pDCs in the epidermis was very low both in PPT and NPT lesions.

Negative results of PPT in patients with AE and sensitizations to pollen allergens were also observed by other authors [44]. It opens the question whether skin or even keratinocyte-specific factors play a role in these mechanisms. This idea is strengthened by the observation of positive PPT reactions in patients without sensitizations, intrinsic or non-AE, which were associated with an influx of IDEC [15]. Furthermore, this observation argues for allergen-specific rather than irritative factors accounting for eczematous reactions to native pollen grains. A question which cannot be answered in our setting of experiments, to some extent because of the low numbers of patients, is whether pollen grains are more competent to induce eczematous reactions compared to the prepared protein extracts for the APT.

In summary, we conclude that pollen grains induce an inflammatory reaction in susceptible individuals with AE. This reaction appears clinically and immunohistochemically similar to the known contact hypersensitivity reaction to nickel but follows a faster kinetics and a biphasic course – IL-5 and IgE in the early and IFN- γ predominance in the late phase. The faster kinetics was also reflected by the rapid occurrence of DC, especially IDEC. Whether the early IL-5 dominance could be in part due to the recently described Th2-polarizing pollen-associated lipid mediators remains to be investigated.

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